



Biopharmaceutical Development Program

Standard Operating Procedure

Title: Quantitation of Bovine IgG Using the Cygnus Inc. ELISA Kit

SOP Number: 22121

Revision Number: 02

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Originator/Date:

Approval/Date:

Approval/Date:

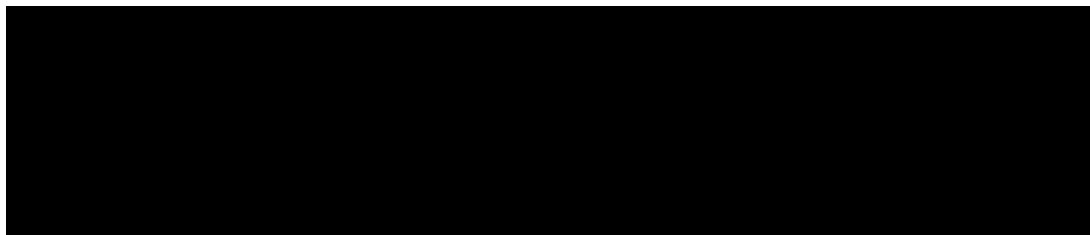


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1.0 Purpose

This Standard Operating Procedure (SOP) describes the use of the Cygnus, Inc. ELISA kit for Bovine IgG quantitation.

2.0 Scope

This SOP applies to PA personnel who will perform the Bovine IgG ELISA.

Note: It is required that new (not previously analyzed) test articles (final product, bulk, or in-process sample) are submitted for R&D testing to determine assay suitability for a particular test article. Conditions necessary for acceptable spike recovery and dilutional linearity must be established prior to GMP testing.

3.0 Authority and Responsibility

- 3.1 The Director, Process Analytics (PA) has the authority to define this procedure.
- 3.2 PA personnel are responsible for performance of this procedure.
- 3.3 PA is responsible for reviewing the data and documentation of the results of this procedure.
- 3.4 Biopharmaceutical Quality Assurance (BQA) is responsible for quality oversight of this procedure.

4.0 Supplies and Reagents

- 4.1 *Bovine IgG* ELISA kit, BDP PN 30126, Cygnus Technologies, Catalog Number F070.
- 4.2 BSA Sample Diluent, BDP PN 30403 Cygnus Technologies, Catalog Number F031A.
- 4.3 Calibrated multi-channel pipettor.
- 4.4 Calibrated pipettors 2-200 μ L and 100-1000 μ L.
- 4.5 Aerosol Barrier Pipet tips, 2-200 μ L, BDP PN 20673 and 1-1000 μ L, BDP PN 20769, VWR, or BDP approved equivalent.
- 4.6 Microtiter plate shaker, VWR Catalog Number 57019-600, or BDP approved equivalent.
- 4.7 Ziploc Bag, BDP PN 20339, or BDP approved equivalent.
- 4.8 Reagent reservoirs, BioWhittaker, BDP PN 20270, or BDP approved equivalent.
- 4.9 Microcentrifuge tubes 0.5 mL, BDP PN 21369, or BDP approved equivalent.
- 4.10 DirectQ water or BDP approved equivalent.
- 4.11 1 Liter Graduated Cylinder for wash solution.
- 4.12 Squirt/wash bottle with the tip cut off.
- 4.13 Low-lint or lint-free absorbent paper, BDP PN 21493, VWR, or BDP approved equivalent.
- 4.14 Labsystems iEMS Microtiter Plate Reader MF with Ascent software version 2.4.2, Model Number 1401, BDP MEF 66160.

5.0 General Comments

- 5.1 Pipetting accuracy and reproducibility are critical for the success of this assay.
- 5.2 Use a new pipette tip for each pipetting procedure (between dilutions).
- 5.3 Avoid contamination of the workspace when handling Bovine IgG standards, ejecting pipette tips, etc. Pipette tips possessing an aerosol barrier must be used.
- 5.4 Good organization and attention to detail are essential to avoid confusion of sample identities and data.

- 5.5 Although the assay is designed to minimize matrix interference, materials such as detergents in high concentration, extremes of pH (< 6.0 and > 8.5) or very high protein concentrations may give erroneous results. A buffer blank is required to be submitted with the sample.

6.0 Preparation

Note: The reagents are located in the 2-8°C refrigerator. Bring all reagents to ambient temperature (up to one hour). All standards, controls, and samples will be assayed in duplicate. The total volume of the samples must take this into account.

6.1 Sample Preparation: Linear Range of the Assay = 0-20 ng/mL.

- 6.1.1 Prepare the test article immediately before use, undiluted and at 1:5 and 1:50 dilution using the BSA sample diluent (see 4.2). If the absorbance results indicate that the value for the 1:50 dilution is greater than 20 ng/mL, then repeat the ELISA assay with appropriately-diluted samples. An example for making dilutions is the following.
- For duplicate measurements of spiked and unspiked diluted test article (a total of four 50 µL aliquots), add 100 µL test article to 400 µL diluent in a microcentrifuge tube to give a 1:5 dilution.
 - A 1:50 dilution can be made up in microcentrifuge tubes by adding 50 µL of the 1:5 diluted test article to 450 µL diluent.
- 6.1.2 Prepare the spiked test article and spiked buffer control. Spiked samples can be prepared as follows.
- A spiked test article can be made up directly in the microtiter strip well. For a spiked concentration of 5 ng/mL, do a 4x dilution into the well using the 20 ng/mL Cygnus, Inc. standard. For example, add 12.5 µL of the 20 ng/mL standard directly into the well containing 37.5 µL of the sample test article prepared in step 6.1.1. Draw the sample and the spike up and down in the pipette tip 2 or 3 times for thorough mixing. Use a new pipette tip with each well.
 - A spiked buffer control sample can be made up directly into the microtiter strip well. For a spiked-buffer control, add 12.5 µL of the 20 ng/mL standard directly into the well containing 37.5 µL of the buffer provided by the requestor. This produces a 5 ng/mL concentration of Bovine IgG in the spiked-buffer control sample. This will need to be done in duplicate. Draw the sample and the spike up and down in the pipette tip 2 or 3 times for thorough mixing.

6.2 Standard Preparation

- 6.2.1 Prepared standards at 0, 0.25, 1, 4, and 20 ng/mL are included in the Cygnus Bovine IgG kit. In order to increase the number of points on the standard curve, at least one more standard, a 2 ng/mL or a 10 ng/mL standard, is prepared fresh immediately before use. For example:

- To make a 2 ng/mL standard, do a 1:1 dilution with the 4 ng/mL standard. Add 25 μ L of the 4 ng/mL standard to 25 μ L of the diluent directly into the well. This will need to be done in duplicate. Draw the sample and the spike up and down in the pipette tip 2 or 3 times for thorough mixing. Use a new pipette tip with each well.
- To make a 10 ng/mL standard, do a 1:1 dilution with the 20 ng/mL standard. Add 25 μ L of the 20 ng/mL standard to 25 μ L of the diluent directly into the microtiter strip well. This will need to be done in duplicate. Draw in the pipette tip 2 or 3 times for thorough mixing. Use a new pipette tip with each well.

6.3 Positive Control Preparation

- 6.3.1 The positive control will be either the 2 ng/mL or 10 ng/mL standard that was prepared in section 6.2.1.

6.4 Wash Solution Preparation

- 6.4.1 Empty the entire contents of the wash solution bottle into a 1 L graduated cylinder. Bring to 1 L with DirectQ water. Log the wash solution in the PA Solutions logbook. Label the solution bottle with the QCB number, initials, date prepared, and expiration date. Refer to **SOP 22702, Solutions Used in Process Analytics**.

7.0 Procedure

- 7.1 Prepare an ELISA Work List (Attachment 1) by labeling the wells where the samples and standards will be placed. Count the microtiter strips needed. Remove the required number of microtiter strips from the kit and place them in the provided frame.
- 7.2 Pipette 50 μ L of **standards, controls and samples** into wells indicated on the ELISA Work List (Attachment 1). All standards, controls and samples must be assayed in at least duplicate.
- 7.3 Pipette 100 μ L of **anti-Bovine IgG:HRP** into each well.
- 7.3.1 Pour contents of **anti-Bovine IgG:HRP** bottle into a new reagent reservoir (4.8).
- 7.3.2 Use a multi-pipettor to fill rows of wells. Get fresh pipette tips with each row that is filled.
- 7.4 Carefully place the plate into a Ziploc bag (4.7).
- 7.5 Transfer to the microtiter plate shaker (4.6) and incubate for 2 hour \pm 2 minutes at a setting of "2" (180 rpm).
- 7.6 Using a manual microtiter plate procedure, wash the plate with at least 350 μ L of diluted wash solution from step 6.4 and remove. Wash a total of 4 times.
- 7.6.1 Remove the liquid from the plate as follows. Grab the plate from the bottom with the thumb in the middle of one side and the fingers on the other side. If the thumb and fingers slightly overlap the tabs on the ends of the middle strip or strips, the operator will usually be able to avoid having any strips fall out of the strip holder.

Holding the plate over the sink, turn the plate upside down just as the operator rapidly accelerates the arm and hand downward. Abruptly stop the arm movement, causing the liquid to be forced from the strips into the sink. Repeat the removal motion a second time.

- 7.6.2 Wash the plate. Use the squirt bottle (4.12) with the narrow portion of the tip cut off to give the largest possible orifice so that the flow will be generous and gentle. Fill all wells with the diluted wash solution provided with the kit (See 6.4). Remove the wash solution as described in steps 7.6.1.

Blot and strike the plate as follows. Immediately blot the upside down plate onto the low-lint absorbent paper (4.13). Move the plate to an unused section of the blotting paper and allow it to drain upside down for 30 seconds. Strike the plate hard 4 times over the unused areas of the paper. Do not be afraid to strike vigorously. Anything short of breaking the strip holder or strips is not too hard.

Repeat the washing, blotting and striking procedure 3 more times for a total of 4 washes. After the last wash, let the plate rest upside down for 60 seconds to drain. Strike the plate again 4-6 times rotating the plate 180° between each strike. This rotation ensures that the ends of the plate receive on average the same energy and impact.

- 7.6.3 Wipe the bottom outside of all wells with clean absorbent paper to remove any liquid from the washing. If the washing technique has been performed correctly, the center of each well should have a small film of liquid (< 1 microliter). If the film is not uniform in terms of the area between the wells, or a significant amount of liquid remains in the circular edge of the wells, then strike the plate more forcefully and repeat step 7.6.3. Wells are now ready to have the substrate added to them.

Do not add substrate near the sink location where the removal and striking have taken place since the washing procedure can generate aerosols that could re-contaminate the wells or substrate.

- 7.7 Pipette 100 µL of the TMB substrate as in steps 7.3.1 - 7.3.2.

Note: If the substrate has a distinct blue color prior to the assay, it may have been contaminated. If this appears to be the case, read 100 µL of substrate plus 100 µL of stop solution against a water blank. If the absorbance is greater than 0.1, it may be necessary to obtain new substrate (substrates are not kit specific) or the sensitivity of the assay may be compromised.

- 7.8 Place the microtiter plate into a new Ziploc bag. Incubate at room temperature for 30 ± 2 minutes.
- 7.9 Remove the plate from the Ziploc bag, and pipette 100 µL of the stop solution as in step 7.3.
- 7.10 Read absorbance at 450/630 nm, blanking on the zero standard. Refer to **SOP 22100, Operation of the Labsystems iEMS Microtiter Plate Reader/Dispenser**, for analysis of the data.

Note: Read the plates within 1 hour after adding the stop solution since the color will fade over time.

8.0 Data Analysis and Acceptable Criteria

After the Ascent Bovine IgG Results (Attachment 2) have been printed out from the ELISA run, the results will need to be manually entered into an Excel Summary Worksheet (Attachment 3). The Summary Worksheet is also in a Microsoft Excel format, and the requested information is transcribed from the report generated by the plate reader and calculated by the Excel application. The acceptable spike recoveries and %CV's are calculated in this Summary Worksheet.

- 8.1 Go to windows "Start." Select programs and click on "Microsoft Excel."
- 8.2 Click on "File" and "Open." Select "Quality Control on "scidata on 'fr-s-bdp-vlan' (S:)."
- 8.3 Click on the "PA" folder. Click on the "ELISA Results" folder. Select the specific ELISA test being analyzed; for example, BSA Template, Murine IgG Template, *E.coli* Host Cell Protein Template etc.
- 8.4 Fill in the top portion of the summary sheet with all appropriate information, including QC number, Analyst, Date, Sample ID, Lot Number, Kit Lot Number and Expiration Date.
- 8.5 Fill in the "Bovine IgG Concentration" section with appropriate measured concentration for replicate 1 and 2 from the calculation column of the Ascent ELISA Results (Attachment 2). The "Corrected Concentration," "Average," and "% CV" will automatically be calculated on the spreadsheet. These numbers can be compared to those on the Ascent ELISA Results. They should be approximately the same depending upon rounding numbers. The "%CV" should be less than 25% for samples > 4 ng/mL.
- 8.6 Fill in the "Expected" and "Found" Positive Control and the "% CV" from the Ascent ELISA Results. The positive control will be one of the standards that the analyst manually made up and included in the standard curve (see step 6.2.1). The "%CV" should be less than 25%. The "Found" positive control should be 100% \pm 30 of what is "Expected."
- 8.7 Fill in the "Found" Spike Buffer Control and the "% CV." The spike buffer control is the buffer that the test article is in spiked with a known amount of standard (see step 6.1.2). The "%CV" should be less than 25%. The "Found" spike control should be 100% \pm 30 of what is "Expected."
- 8.8 Fill in replicates 1 and 2 for each dilution with the "Spiked Test Article Concentrations." These numbers are found on the Ascent ELISA Results under the "Calc." column for the spiked samples. These numbers are not corrected for dilutions. The "Average" and "% CV" will automatically be calculated and should be approximately the same as on the Ascent ELISA Results. The % CV should be less than 25%.
- 8.9 The test article must exhibit dilution-corrected analytes concentrations that vary no more than \pm 50% between dilutions. Avoid consideration of dilutional data where the assay value before dilution correction falls below two times the LOQ of the assay (1 ng/mL for the Bovine IgG assay). The first dilution-corrected concentration exhibiting dilutional linearity is reported. In the example below, the reported result would be 50 ng/mL.

Sample Dilution	Dilution-Corrected Value (ng/mL)	Percent Change in Concentration from Previous Dilution
Neat	100	N/A
1:5	50	50
1:25	45	90
1:50	47	104

8.10 If the criteria in sections 8.8 and 8.9 are not both met the assay is invalid.

9.0 Documentation

- 9.1 Print the Excel Summary Worksheet (Attachment 3), sign and date the worksheet and attach it to the QC Test Request Form with a copy of the Ascent Bovine IgG ELISA Results (Attachment 2).
- 9.2 The lowest dilution with the acceptable criteria listed above can be recorded on the QC Test Request Form.

Note: If the results are less than the lowest standard, it is recorded as <1 ng/mL.

- 9.3 Record all solution preparation on Form 22121-01, Bovine IgG ELISA Preparation (Attachment 4) and include with the QC Test Request Form.

10.0 References and Related Documents

- 10.1 **SOP 22100** *Operation of the Labsystems iEMS Microtiter Plate Reader/Dispenser*
- 10.2 **SOP 22702** *Solutions Used in Process Analytics*
- 10.3 Cygnus Technologies Bovine IgG Kit Instructions (Attachment 5).
- 10.4 Cygnus Suggested Manual Microtiter plate Washing Procedure.

11.0 Attachments

- 11.1 **Attachment 1** Sample ELISA Work List
- 11.2 **Attachment 2** Sample Ascent Bovine IgG ELISA Results
- 11.3 **Attachment 3** Sample EXCEL Summary Worksheet
- 11.4 **Attachment 4** Form 22121-01, Bovine IgG ELISA Preparation
- 11.5 **Attachment 5** Cygnus Technologies Inc. Bovine IgG ELISA Kit Insert

Attachment 1 Sample ELISA Work List

H	G	F	E	D	C	B	A	↑
20 ng/ml Standard	10 ng/ml Standard	4 ng/ml Standard	2 ng/ml Standard	1 ng/ml Standard	0.25 ng/ml Standard	0 ng/ml Standard	BLANK 0ng/ml Std	1
20 ng/ml Standard	10 ng/ml Standard	4 ng/ml Standard	2 ng/ml Standard	1 ng/ml Standard	0.25 ng/ml Standard	0 ng/ml Standard	BLANK 0ng/ml Std	2
SPIKED SAMPLE 1:50 dilution	SAMPLE 1:50 dilution	SPIKED SAMPLE 1:5 dilution	SAMPLE 1:5 dilution	SPIKED SAMPLE Undiluted	SAMPLE Undiluted	SPIKED Buffer CONTROL	Buffer CONTROL	3
SPIKED SAMPLE 1:50 dilution	SAMPLE 1:50 dilution	SPIKED SAMPLE 1:5 dilution	SAMPLE 1:5 dilution	SPIKED SAMPLE Undiluted	SAMPLE Undiluted	SPIKED Buffer CONTROL	Buffer CONTROL	4
								5
								6
								7
								8
								9
								10
								11
								12

Assay:

Sample ID:

Date:

Analyst:

Attachment 2

Sample Ascent Bovine IgG ELISA Results

Ascent Software

Curve Fit1

6/15/04 4:46 PM

Session: C:\ASCENT\050604.SEE
 Instrument: EMS Reader MF V2.9-0D
 User name: XXXXXXXXXX
 Started at: 5/6/04 4:45:22PM
 Actual temperature: Amb.temp.

Layout map for calibrators Sheet: Measure1, Assay: Assay1 and for samples Sheet: Measure1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10
A	Blank	Blank			pool	pool	abx	abx	Q	Q
B	Cal1	Cal1			spool	spool	sabx	sabx	sQ	sQ
C	Cal2	Cal2			pool	pool	A	A	Q	Q
D	Cal3	Cal3			spool	spool	sA	sA	sQ	sQ
E	Cal4	Cal4	nc	nc	abx	abx	A	A	Q	Q
F	Cal5	Cal5	snc	snc	sabx	sabx	sA	sA	sQ	sQ
G	Cal6	Cal6	pool	pool	abx	abx	A	A		
H	Cal7	Cal7	spool	spool	sabx	sabx	sA	sA		

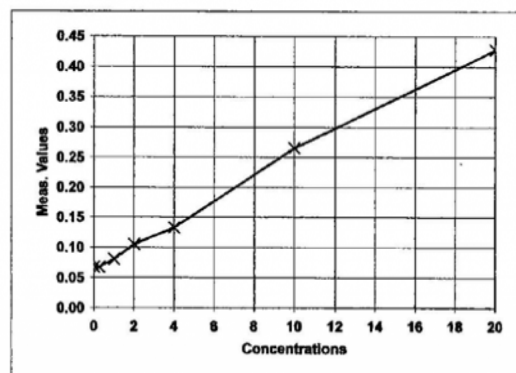
Source data for calibrators Sheet: Measure1, Assay: Assay1 and for samples Sheet: Measure1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10
A	0.065	0.060			0.257	0.273	0.064	0.067	0.246	0.344
B	0.065	0.069			0.312	0.285	0.119	0.126	0.290	0.260
C	0.071	0.062			0.101	0.109	0.753	0.775	0.156	0.145
D	0.080	0.080			0.171	0.162	0.658	0.673	0.236	0.216
E	0.102	0.107	0.066	0.066	0.255	0.205	0.152	0.150	0.075	0.090
F	0.133	0.131	0.125	0.127	0.271	0.266	0.229	0.206	0.137	0.145
G	0.261	0.269	0.861	0.937	0.111	0.116	0.072	0.069		
H	0.413	0.441	0.788	0.727	0.179	0.177	0.139	0.135		

Sheet: Measure1, Assay: Assay1

Name	Meas.	Conc.
Cal1	0.065 0.069 0.067	0.00
Cal2	0.071 0.062 0.067	0.25
Cal3	0.080 0.080 0.080	1.00
Cal4	0.102 0.107 0.105	2.00
Cal5	0.133 0.131	

	Conc.	Meas.	CalcConc.	Residual
Cal1	0	0.067	0.27778	-0.27778
Cal2	0.25	0.067	0.25	1.3878e-16
Cal3	1	0.080	1	2.2204e-16
Cal4	2	0.105	2	0
Cal5	4	0.132	4	0
Cal6	10	0.265	10	0
Cal7	20	0.427	20	0



Attachment 3 Sample EXCEL Summary Worksheet

Summary of Bovine IgG ELISA Results

QC Number: QC-00000 Analyst: XXXXXXXXXX Date: 4/19/2010

Sample ID: Project Name: Lot #: LXX00000

Kit Lot#: 91110 Expiration Date: 1/31/2011

Bovine IgG Concentration (ng/mL)						
Acceptable Criteria: %CV <25% for samples >4ng/mL						
Dilution Factor	Measured Concentration		Corrected Concentration		Average	%CV
	Replicate 1	Replicate 2	Replicate 1	Replicate 2		
1.00	195.02	190.32	195.02	190.32	192.67	1.72
5.00	40.04	46.32	200.20	231.60	215.90	10.28
10.00	21.26	19.52	212.60	195.20	203.90	6.03
20.00	11.68	10.58	233.60	211.60	222.60	6.99
40.00	5.33	4.65	213.20	186.00	199.60	9.64
Final Result:					192.67 ng/mL	
Corrected Concentration = Measured Concentration x dilution factor						
*n/c = not calculated						

Calculated Concentration of "Bovine IgG Spiking Solution" (ng/mL): 20.0

Correlation Coefficient of Standard Curve: point to point

Acceptable Criteria: %Recovery (100% \pm 30) and %CV <25%

	Expected	Found	%Recovery	%CV
Avg. Positive Control (ng/mL):	10.00	10.01	100.10%	7.88
Spiked Buffer Control (ng/mL)	5.00	4.14	82.80%	8.15

Dilutional Linearity

Dilution Factor	Average Dilution Corrected Concentration	Percent Change in Dilution Corrected Concentration from Previous Value
1.00	192.67	N/A
5.00	215.90	112
10.00	203.90	94
20.00	222.60	109
40.00	199.60	90

Acceptability Criteria: Dilution Corrected Concentration 50 - 150% of Previous Value

Attachment 3 (Continued)

Spiked Test Article Concentrations (ng/mL; not corrected for dilution):					
Acceptable Criteria: %CV <25%					
	<u>Dilution</u> <u>Factor</u>	<u>Replicate 1</u>	<u>Replicate 2</u>	<u>Average</u>	<u>%CV</u>
	1	210.65	206.54	208.60	1.39
	5	85.66	86.34	86.00	0.56
	10	56.30	60.54	58.42	5.13
	20	60.48	57.89	59.19	3.09
	40	56.23	52.47	54.35	4.89

Percent Spike Recovery of Test Article:					
Acceptable Criteria: Average Recovery (100% ± 30) and %CV <25%					
	<u>Dilution</u> <u>Factor</u>	<u>Replicate 1</u>	<u>Replicate 2</u>	<u>Average</u>	<u>%CV</u>
	1	1287.70	1276.00	1281.85	0.65
	5	1112.60	1032.00	1072.30	5.32
	10	807.10	918.00	862.55	9.09
	20	1034.40	999.10	1016.75	2.45
	40	1044.65	979.65	1012.15	4.54

Percent Spike Recovery = $\frac{(((\text{Spiked Conc} \times 0.050 \text{ mL}) - (\text{Unspiked Conc} \times 0.0375 \text{ mL})) / (\text{Spike Conc} \times 0.0125 \text{ mL}))}{1} \times 100\%$

Analyst/Date: _____

Reviewed By/Date: _____

Attachment 4

NCI-Frederick
Form No.: 22121-01
SOP No.: 22121
Revision 02:

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Bovine IgG ELISA PREPARATION**QC Number:** _____ **Operator:** _____ **Date:** _____

Plate Reader MEF Number: _____ Calibration Due Date: _____

Diluent:

Name: _____ Lot# _____ Exp. Date: _____

Test sample Dilutions:**Test Sample #1**

Name: _____ Appearance: _____

Lot # _____

Protein Concentration: _____ mg/mL

Initial dilution : _____

Volume of diluent: _____ μ LVolume of test sample: _____ μ L

Second dilution : _____

Volume of diluent: _____ μ LVolume of initial diluted test sample _____ μ L

Fourth dilution: _____

Volume of diluent: _____ μ LVolume of third diluted test sample _____ μ L

Third Dilution _____

Volume of Diluent: _____ μ LVolume of Second Diluted sample _____ μ L

Fifth dilution: _____

Volume of diluent: _____ μ LVolume of fourth diluted test sample _____ μ L**Test sample #2**

Name: _____ Appearance: _____

Lot # _____

Protein Concentration: _____ mg/mL

Initial dilution : _____

Volume of diluent: _____ μ LVolume of test sample: _____ μ L

Second dilution : _____

Volume of diluent: _____ μ LVolume of initial diluted test sample _____ μ L

Fourth dilution: _____

Volume of diluent: _____ μ LVolume of third diluted test sample _____ μ L

Third Dilution _____

Volume of Diluent: _____ μ LVolume of Second Diluted sample _____ μ L

Fifth dilution: _____

Volume of diluent: _____ μ LVolume of fourth diluted test sample _____ μ L**Test sample #3**

Name: _____ Appearance: _____

Lot # _____

Protein Concentration: _____ mg/mL

Initial dilution : _____

Volume of diluent: _____ μ LVolume of test sample: _____ μ L

Attachment 4 (Continued)

NCI-Frederick
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Second dilution: _____
Volume of diluent: _____ μL
Volume of initial diluted test sample _____ μL

Fourth dilution: _____
Volume of diluent: _____ μL
Volume of third diluted test sample _____ μL

Third Dilution _____
Volume of Diluent: _____ μL
Volume of Second Diluted sample _____ μL

Fifth dilution: _____
Volume of diluent: _____ μL
Volume of fourth diluted test sample _____ μL

Positive Control

Preparation of Positive Control
Concentration: _____ ng/mL
Std: _____ μL
Sample Dilution Buffer: _____ μL

Spiked Buffer Control

Preparation of Spiked Buffer Control
Concentration: _____ ng/mL
Std: _____ μL
Sample Buffer: _____ μL

Template Procedure**anti-Bovine IgG:HRP Antibody**

Lot# _____
Expiration Date: _____
Volume of antibody added to wells: _____
Incubation Time: _____
Washing times: _____

Substrate

Lot # _____
Expiration Date: _____
Volume of substrate added to wells: _____
Incubation Time: _____

Stop solution

Lot # _____
Expiration Date: _____
Volume of stop added to wells: _____

Data Storage

File Name: _____

Performed by/Date: _____

Reviewed by/Date: _____

Attachment 5 Cygnus Technologies Inc. Bovine IgG ELISA Kit Insert



Bovine IgG Assay

Immunoenzymetric Assay for the Measurement of Bovine IgG Catalog # F070

Intended Use

This kit is intended for use in quantitating bovine IgG. The kit is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals.

Summary and Explanation

The manufacture of products by various biotechnological processes such as cell or tissue culture can result in residual contamination of the desired product by components used in the culture media. Many formulations of media contain either bovine serum or other more purified fractions of serum proteins such as albumin or transferrin which may contain varying amounts of bovine IgG. When the intended product may be used as a therapeutic agent in humans or animals the product should be highly purified to avoid potential health risks or other problems that might result from trace contaminants. Efforts to reduce trace media contamination to the lowest level practical through optimal process design, validation, and final product testing require a highly sensitive and reliable analytical method. The *Cygnus Technologies* Bovine IgG assay is designed to provide a simple to use, precise, and highly sensitive method to detect contamination to less than 250pg/mL. As such, this kit can be used as a tool to aid in optimal purification process development and in routine quality control of in-process streams as well as final product.

Principle of the Procedure

The Bovine IgG assay is a two-site immunoenzymetric assay. Samples containing Bovine IgG are reacted in microtiter strips coated with an affinity purified capture antibody. A second HRP labeled anti-Bovine IgG antibody is reacted, forming a sandwich complex of solid phase antibody-Bovine IgG-HRP labeled antibody. After a wash step removes any unbound reactants, the strips are then reacted with TMB substrate. Followed by the addition of a stop solution changing the color from blue to yellow. The amount of hydrolyzed substrate is read on a microtiter plate reader and will be directly proportional to the concentration of bovine IgG present in the sample. Accurate quantitation is achieved by comparing the signal of unknowns to bovine IgG standards assayed at the same time.

Reagents & Materials Provided

Component	Product #
Anti-bovine IgG:HRP Sheep antibody (H&L chain specific) conjugated to horseradish peroxidase with preservative. 1x12mL	F071
Anti-bovine IgG coated microtiter strips 12x8 well strips in a bag with desiccant	F072*
Bovine IgG Standards Bovine IgG in a protein matrix with preservative. Standards at 0, 0.25, 1, 4, and 20 ng/mL. 1mL/vial	F073
Stop Solution 0.5N sulfuric acid. 1x12mL	F006
TMB Substrate 3,3',5,5' Tetramethylbenzidine. 1x12mL	F005
Wash Concentrate (20X) Tris buffered saline with preservative. 1x50mL	F004

*All components can be purchased separately except # F072.

Storage & Stability

* All reagents should be stored at 2°C to 8°C for stability until the expiration date printed.

* The substrate reagent should not be used if its stopped absorbance at 450nm is greater than 0.1.

* Reconstituted wash solution is stable until the expiration date of the kit.

Materials & Equipment Required But Not Provided

Microtiter plate reader spectrophotometer with dual wavelength capability at 450 & 650nm. (If your plate reader does not provide dual wavelength analysis you may read at just the 450nm wavelength.)

Pipettors - 50µL and 100µL
Repeating or multichannel pipettor - 100µL
Microtiter plate rotator (150 - 200 rpm)
Sample Diluent (recommended Cat # F031A)
Distilled water
1 liter wash bottle for diluted wash solution

Attachment 5 (Continued)

Precautions

- * For Research or Manufacturing use only.
- * Stop reagent is 0.5N H₂SO₄. Avoid contact with eyes, skin, and clothing. At the concentrations used in this kit, none of the other reagents are believed to be harmful.
- * This kit should only be used by qualified technicians.

Preparation of Reagents

- * Bring all reagents to room temperature.
- * Dilute wash concentrate to 1 liter in distilled water, label with kit lot and expiration date, and store at 4°C.

Procedural Notes

1. Complete washing of the plates to remove excess unreacted reagents is essential to good assay reproducibility and sensitivity. We advise against the use of automated or other manual operated vacuum aspiration devices for washing plates as these may result in lower specific absorbances, higher non-specific absorbance, and more variable precision. The manual wash procedure described below generally provides lower backgrounds, higher specific absorbance, and better precision. If duplicate CVs are poor or if the absorbance of the "0" standard minus a substrate blank is greater than 0.2, evaluate plate washing procedure for proper performance.
2. This kit is a very sensitive assay for bIgG (< 150pg/mL). Since bovine serum and other bovine protein products are common reagents in many laboratories and are often used at relatively high concentrations it is very important to use extreme care to avoid contamination of any of the components in this kit with external sources of bIgG or other bovine protein products. bIgG contamination will manifest itself as either high assay background, poor precision, or unexpected results.
3. Dilution of samples will be required for samples >20ng/mL. The diluent used should be compatible with accurate recovery. The preferred diluent is our Cat. # F031A available in 100mL, 500mL, or 1 liter bottles. This is the same material used to prepare the kit standards. As the sample is diluted in F031A its matrix begins to approach that of the standards thus reducing any inaccuracies caused by dilutional artifacts. Other prospective diluents should be validated in the assay to demonstrate that they do not give elevated background and are not contaminated with bIgG or other bovine proteins. The diluent should also give acceptable recovery when spiked with known quantities of bIgG.
4. High Dose Hook Effect may be observed in samples with very high concentrations of bIgG. Samples greater than 2µg/mL may give absorbances less than the 20ng/mL standard. If a hook effect is possible samples should also be assayed diluted. If the dilution corrected concentration of the diluted sample is greater than the undiluted sample this may be indicative of the hook effect.

Limitations

- * The antibodies used in this kit may cross react with bovine IgM. Cross reactivity with other species has not been extensively investigated.
- * Certain sample matrices may interfere in this assay. Although the assay is designed to minimize matrix interference, materials such as detergents in high concentration, extremes of pH (<6.0 and >8.5) or very high protein concentrations may give erroneous results. It is recommended to test the sample matrix for interference by diluting the 20ng/mL standard 1 part to 3 parts of the matrix that does not contain any bIgG. This diluted standard when assayed as an unknown should give a value of 5ng/mL \pm 20%. In cases where bIgG levels in the sample will allow for sample dilution, such dilution will often overcome sample matrix interference. Consult Cygnus Technologies Technical Service Department for advice on how to quantitate the assay in problematic matrices.

Assay Protocol

- * The assay is very robust such that assay variables like incubation times, sample size, and other sequential incubation schemes can be altered to manipulate assay performance for more sensitivity, increased upper analytical range, or reduced sample matrix interference. Before modifying the protocol from what is recommended, users are advised to contact our technical services for input on the best way to achieve your desired goals.
- * The protocols specify use of an approved microtiter plate shaker for the immunological step. These can be purchased from most laboratory supply companies. Alternatively you can purchase an approved, pre-calibrated shaker directly from Cygnus Technologies. If you do not have such a device, it is possible to incubate the plate without shaking however it will be necessary to extend the immunological incubation step in the plate by about 60 minutes in order to achieve comparable results to the shaking protocol. **Do not shake during the 30-minute substrate incubation step as this may result in higher backgrounds and worse precision.**
- * Avoid the assay of samples containing Sodium Azide, (NaN₃) which will destroy the HRP activity of the conjugate and could result in the under-estimation of bIgG levels in that sample.
- * Bring all reagents to room temperature.
- * Set-up plate spectrophotometer to read dual wavelength at 450nm for the test wavelength, and 650nm for the reference wavelength. Blank the instrument using the zero standard wells after assay completion.
- * All standards, controls and samples should be assayed in duplicate. Samples that could contain very high levels of bIgG above the 20ng/mL standard or in the "Hook" region of this assay should also be assayed diluted. Recommended diluent is Cygnus Technologies Cat # F031A. Avoid the use of diluents which contain NaN₃ or could be contaminated with trace levels of bIgG or other bovine proteins.

Attachment 5 (Continued)

* Thorough washing is essential to proper performance of this assay. Automated plate washing systems or other vacuum aspiration devices are not recommended. The manual method described in the assay protocol is preferred for best precision, sensitivity and accuracy. A more detailed discussion of this procedure can be obtained from our Technical Services Department or on our web site.

* Maintain a repetitive timing sequence from well to well for all assay steps to insure that all incubation times are the same for each well.

* Make a work list for each assay to identify the location of each standard control and sample.

* If the substrate has a distinct blue color prior to the assay it may have been contaminated. If this appears to be the case, read 100 μ L of substrate plus 100 μ L of Stop Solution against a water blank. If the absorbance is greater than 0.1 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.

* Plates should be read within 30 min. after adding stop since color will fade over time.

Assay Protocol

1. Pipette 50 μ L of standards, controls and samples into wells indicated on work list.
2. Pipette 100 μ L of anti-bovine IgG:HRP (#F071) into each well.
3. Cover & incubate on rotator at ~ 180rpm for 2 hours at room temperature, 24°C \pm 4°.
4. Dump contents of wells into waste or gently aspirate with a pipettor. Blot and vigorously bang out residual liquid over absorbent paper. Fill wells generously with diluted wash solution by flooding well from a squirt bottle or by pipetting in ~350 μ L. Dump and bang again. Repeat for a total of 4 washes. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. Do not allow wash solution to remain in wells for longer than a few seconds. Do not allow wells to dry before adding TMB substrate.
5. Pipette 100 μ L of TMB substrate (#F005).
6. Incubate at room temperature for 30 minutes. DO NOT SHAKE.
7. Pipette 100 μ L of Stop Solution (#F006).
8. Read absorbance at 450/650nm blanking on the Zero standard.

Quality Control

* Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples greater than 1ng/mL. CVs for samples <1ng/mL may be greater than 10%.

* For optimal performance the absorbance of the substrate when blanked against water should be <0.1.

* It is recommended that each laboratory assay appropriate quality control samples in each run to insure that all reagents and procedures are correct.

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL. This data reduction may be performed through computer methods using curve fitting routines such as point-to-point, spline, or 4 parameter logistic fit. **Do not use linear regression analysis to interpolate values for samples as this may lead to significant inaccuracies!** Data may also be manually reduced by plotting the absorbance values of the standard on the y-axis versus concentration on the x-axis and drawing a smooth point-to-point line. Absorbances of samples are then interpolated from this standard curve.

Example Data

Well #	Contents	Abs. at 450nm	Mean Abs.	ng/mL bigG
1A	Zero Std	0.000		
1B	Zero Std	0.002	0.001	
1C	0.25ng/mL	0.035		
1D	0.25ng/mL	0.031	0.033	
1E	1ng/mL	0.124		
1F	1ng/mL	0.123	0.124	
1G	4ng/mL	0.467		
1H	4ng/mL	0.485	0.476	
2A	20ng/mL	1.785		
2B	20ng/mL	1.705	1.745	
2C	sample 1	0.005		
2D	sample 1	0.010	0.008	<0.1ng
2E	sample 2	0.127		
2F	sample 2	0.123	0.125	1ng

Performance Characteristics

Cygnus Technologies has validated this assay by conventional criteria as indicated below. A more detailed copy of this "Validation Summary" report can be obtained by request. This validation is generic in nature and is intended to supplement but not replace certain user and product specific qualification and validation that should be performed by each laboratory. At a minimum each laboratory is urged to perform a spike and recovery study in their sample types. In addition, any of your samples types containing bigG within or above the analytical range of this assay should be evaluated for dilutional linearity to insure that the assay is accurate and has sufficient antibody excess for your particular bigG samples. Each laboratory and technician should also demonstrate competency in the assay by performing a precision study similar to that described below. A more detailed discussion of recommended user validation protocols can be obtained by contacting our Technical Services Department or at our web site.

Attachment 5 (Continued)

Sensitivity

The lower limit of detection (LOD) is defined as that concentration corresponding to a signal two standard deviations above the mean of 10 replicates of the zero standard. LOD is ~100 pg/mL in the recommended protocol. The lower limit of quantitation (LOQ) is defined as the lowest concentration, where concentration coefficients of variation (CVs) are <20%. The LOQ is ~150 pg/mL.

Precision

Precision is defined as the percent coefficient of variation (%CV). This is calculated by dividing the standard deviation by the mean value for a number of replicate determinations of two different control samples in the low and high concentration range of the assay. Both intra and inter-assay (n=5 assays) precision were determined on 2 pools with low (~1ng/mL) and high concentrations (~5ng/mL).

Intra-assay			Inter-assay		
# of tests	Mean ng/mL	%CV	# of assays	Mean ng/mL	%CV
20	1.1	7.2	5	1.1	7.8
20	5.2	4.3	5	5.0	6.6

Specificity/Cross-Reactivity

In sandwich ELISA cross reactivity can manifest itself either as a false increase in bIgG (positive cross reactivity) or as a false decrease in bIgG (negative cross reactivity) when bIgG present in the sample competes with the cross reactant for the kit antibodies. Mouse IgG and human IgG1, IgG2a, IgG2b, and IgA were tested at 2 mg/mL and found to have no cross reactivity in this assay. All matrices should be evaluated by the user for the possibility of cross reactivity.

Recovery/ Interference Studies

Various buffer matrices were evaluated by adding known amounts of bIgG. Because this assay is designed to minimize matrix interference most of these buffers yielded acceptable recovery defined as between 80-120%. In general, extremes in pH (<6.0 and >8.5), high salt concentrations, as well as certain detergents can cause under-recovery. Some product proteins in high concentration may also interfere in the accurate measurement of bIgG. Each user should validate that their sample matrices yield accurate recovery. Such an experiment can be easily performed by diluting the 20ng/mL standard provided with this kit into the sample matrix in question. For example, add 1 part of the 20ng/mL standard to 3 parts of the matrix containing no or very low bIgG contaminants. This diluted standard when assayed as an unknown should give a value of 5 +/- 20%. Consult Cygnus Technologies Technical Service Department for advice on how to quantitate the assay in problematic matrices.

Hook Capacity

Increasing concentrations of HCPs > 20 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration that can give an absorbance reading less than the 20ng/mL standard, was 2 µg/mL.

Ordering Information/ Customer Service

To place an order or to obtain additional product information contact Cygnus Technologies:

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